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Clinicopathological features of genetically confirmed Danon disease

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Abstract—*Background*: Danon disease is due to primary deficiency of lysosome-associated membrane protein-2. *Objective*: To define the clinicopathologic features of Danon disease. *Methods*: The features of 20 affected men and 18 affected women in 13 families with genetically confirmed Danon disease were reviewed. *Results*: All patients had cardiomyopathy, 18 of 20 male patients (90%) and 6 of 18 female patients (33%) had skeletal myopathy, and 14 of 20 male patients (70%) and one of 18 female patients (6%) had mental retardation. Men were affected before age 20 years whereas most affected women developed cardiomyopathy in adulthood. Muscle histology revealed basophilic vacuoles that contain acid phosphatase–positive material within membranes that lack lysosome-associated membrane protein-2. Heart transplantation is the most effective treatment for the otherwise lethal cardiomyopathy. *Conclusions*: Danon disease is an X-linked dominant multisystem disorder affecting predominantly cardiac and skeletal muscles.

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Danon disease, an X-linked cardioskeletal myopathy originally reported as "lysosomal glycogen storage disease with normal acid maltase,"[1] is caused by primary deficiency of lysosome-associated membrane protein-2 (LAMP-2), a major lysosomal membrane protein.[2] Together with its paralogous counterpart LAMP-1, LAMP-2 is a highly glycosylated protein coating the inner side of the lysosomal membrane. The *LAMP-2* gene is located on Xq24.[3] LAMP-2 is thought to protect the lysosomal membrane from proteolytic enzymes within lysosomes and to act as a receptor for proteins to be imported into lysosomes.[4] However, the precise functional role of LAMP-2 is still controversial.

Danon disease is characterized clinically by the triad of cardiomyopathy, myopathy, and mental re-

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Table 1 *Summary of 13 families with Danon disease*

Family	Ethnic background	Site of *LAMP-2* gene mutation	Affected men	Affected women	Study
1	Japanese	E9b	Proband, 1 cousin	Mother, 1 sister	9, 12
2	Japanese	E4	Proband	—	16
3	Japanese	I6*	Proband	Mother	3, 11
4	Italian	E8	Proband, 1 nephew	Mother, 1 sister	10
5	Afro-American	E8	Proband	Mother, 1 grandmother	
6	Japanese	I5	Proband	—	
7	Afro-American	I5	Proband	Mother, 2 sisters	7
8	Anglo-Saxon	I5	Proband, 1 brother	Mother	4
9	Japanese	I5/E6 junction	Proband	Mother	
10	Greek	E1	Proband, 2 cousins	Mother, 1 aunt	14
11	Japanese	E7	Proband	1 Sister	
12	Spanish	E2	Proband, 2 cousins	Mother, 1 grandmother, 1 aunt	
13	Italian	E4	Proband	—	

* Exon skipping mutation.

tardation.[1,5,6] Muscle biopsy reveals small autophagic vacuoles in muscle fibers. Some of these vacuoles have features of plasma membrane.[7] Diagnosis had been based on these clinical and histologic features in addition to normal acid maltase activity in skeletal muscle. However, disorders reported as "lysosomal glycogen storage disease with normal acid maltase" are genetically heterogeneous. In fact, two patients reported to have an infantile form of Danon disease did not have LAMP-2 deficiency.[8]

To better delineate the clinicopathologic features of Danon disease, we studied 38 patients from 13 families with genetically confirmed Danon disease.

Materials and methods. *Patients.* We reviewed the clinicopathologic features of 20 affected men and 18 affected women from 13 families with genetically confirmed Danon disease. Immunohistochemistry, Western blot analyses, or both confirmed LAMP-2 deficiency in all specimens. Clinical manifestations of seven of the 13 families were reported previously.[1,5,6,9-14] Statistical values are expressed as mean ± SD.

Sequence analysis. The open reading frame of the *LAMP-2* gene consists of nine exons. Human exon 9 exists in two forms, exon 9a and 9b, that are alternatively spliced and processed into two isoforms, *LAMP-2a* and *LAMP-2b*.[15] We sequenced the entire coding region, including both exons 9a and 9b, and the exon/intron junctions of the *LAMP-2* gene as described.[2]

Muscle biopsy. Muscle biopsy was performed in all probands. Biopsy specimens were either frozen in liquid nitrogen–cooled isopentane for histochemistry or fixed with glutaraldehyde for electron microscopy. Transverse serial frozen sections of 10-μm thickness were stained with hematoxylin and eosin, modified Gomori trichrome, and a battery of histochemical methods.

In addition, we performed indirect immunofluorescence staining on 6-μm cryosections of muscle using mouse monoclonal antibodies according to methods described previously.[16] These sections were incubated at 37 °C for 2 hours with the primary mouse IgG antibodies against LAMP-2 (H4B4, Developmental Studies Hybridoma Bank, Iowa City, IA), the C-terminal of dystrophin (NCL-DYS2, Novocastra, Newcastle Upon Tyne, UK), laminin α2 (NCL-MEROSIN, Novocastra), and α-sarcoglycan (NCL-α-SARC, Novocastra). They were subsequently incubated at 37 °C for 1 hour with a secondary antibody fluorescein isothiocyanate–labeled goat F(ab')2 antimouse IgG (M102, Leinco Technology, St. Louis, MO). All sections were examined by fluorescence microscopy. Control specimens were obtained from 10 patients with morphologically normal muscle.

For electron microscopy, biopsy specimens were fixed in buffered 2% isotonic glutaraldehyde at pH 7.4, postfixed in osmium tetroxide, and embedded in epoxide resin. Ultrathin sections were stained with uranyl acetate and lead nitrate and examined with an H-7000 electron microscope (Hitachi). When sufficient tissues was available, we also performed Western blot analysis.

Results. *LAMP-2 gene mutations.* We identified *LAMP-2* mutations in all of the probands and affected family members from whom samples were available (table 1).[5] All mutations were either nonsense or frame-shift mutations that are predicted to cause truncation of the protein, except for the exon 6 skipping mutation in Family 3.[5]

Protein analysis. On immunohistochemical analysis, LAMP-2 staining was completely absent in muscle of the probands. By Western blot analysis, LAMP-2 was undetectable except for the proband in Family 1 who showed a small amount of LAMP-2 protein, as reported previously.[5]

Clinical features. The clinical features in 20 male patients with Danon disease are summarized in table 2. All 13 probands were male. The two most common features were cardiomyopathy and myopathy; mental retardation was present in 14 of the 20 male patients.

Ages at onset in the 20 male patients varied from 10 months to 19 years. Onset may be earlier but may go undetected because of the subacute nature and slow pro-

Table 2 *Clinical features of 20 male and 18 female patients with Danon disease*

Characteristics	Male	Female
Subjects, n	20	18
Age, y, mean ± SD	17 ± 7 (n = 20)	38 ± 12 (n = 14)
Age at onset, n		
Infantile	4	
Childhood	11	
Second decade	5	
Age at death, y, mean ± SD	19 ± 6 (n = 7)	40 ± 7 (n = 6)
Cause of death, n (%)		
Cardiac failure	7/7 (100)	6/6 (100)
Myopathy, n (%)	18/20 (90)	6/18 (33)
Muscle weakness	16/20 (80)	6/18 (33)
Fatigability only	2/20 (10)	0/18 (0)
Cardiomyopathy, n (%)	20/20 (100)	18/18 (100)
Hypertrophic	16/19 (84)	2/7 (29)
Dilated	2/19 (11)	5/7 (71)
Mixed	1/19 (5)	
Mental retardation, n (%)	14/20 (70)	1/18 (6)
Elevated CK, n (%)	18/18 (100)	5/8 (63)
Serum CK (IU/L), mean ± SD	1574 ± 790 (n = 18)	
Serum AST (IU/L), mean ± SD	382 ± 234 (n = 12)	
Serum ALT (IU/L), mean ± SD	344 ± 199 (n = 11)	
Serum LDH (IU/L), mean ± SD	1874 ± 935 (n = 11)	
Serum aldolase (IU/L), mean ± SD	17.4 ± 8.0 (n = 9)	
Abnormal EKG, n (%)	17/17 (100)	10/10 (100)
Age at abnormal EKG, y, mean ± SD	13 ± 5 (n = 10)	
Abnormal echocardiogram, n (%)	19/19 (100)	3/3 (100)
Pacemaker, n (%)	6/20 (30)	3/18 (17)
Heart transplantation, n (%)	1/20 (5)	2/18 (11)
Myogenic EMG, n (%)	10/10 (100)	
Hepatomegaly, n (%)	5/14 (36)	
Splenomegaly, n (%)	1/13 (8)	
Foot deformity, n (%)	3/8 (38)	
Delayed milestone, n (%)	3/8 (38)	
Abnormal EEG, n (%)	2/7 (29)	
Manifesting mother, n (%)	14/20 (70)	6/18 (33)

CK = creatine kinase; AST = aspartate transaminase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; EMG = electromyography.

gression of the disease. For example, two male patients were first identified when isolated increases in serum creatine kinase were noted, and two others because they had abnormal EKG findings preceding cardiac symptoms. Only one male patient developed dyspnea in the infantile period; most commonly onset is of childhood onset. All patients were born through normal pregnancy and delivery. Delayed milestones with mild mental retardation were observed in three male patients. Five male patients ran slower than their peers during childhood. The mean age at death was 19 years, with a range of 12 to 29 years; all died of cardiac failure or sudden cardiac arrest. No male patients survived beyond age 29 years except for a 34-year-old man in Family 1. He had mild cardiomyopathy but reported no cardiac symptoms.

All 20 male patients had cardiomyopathy. Most had hypertrophic cardiomyopathy, but two had dilated cardiomyopathy, and one had mixed features of hypertrophic and dilated cardiomyopathy. Permanent pacemakers were inserted in six male patients (30%). Heart transplantation was performed in one male patient (5%).

Myopathy was usually mild and was observed in 17 of 20 male patients (85%). The main symptoms were mild proximal limb and neck muscle weakness. All male patients remained ambulatory. Two male patients without weakness had only premature fatigability without fixed weakness. When present, muscle atrophy was mild. Only three male patients had obvious muscle wasting.

Mild mental retardation was observed in 14 male patients (70%). IQ was reduced to a range of 60 to 91 in five male patients in whom formal cognitive tests were performed.

On physical examination, hepatomegaly was observed in five of 14 male patients (36%) and splenomegaly in one of 13 male patients (8%). Foot deformities, such as pes cavus, was seen in three of eight male patients (38%).

Laboratory tests showed that serum creatine kinase and aldolase were increased five- to 10-fold above upper limits of normal. Creative kinase values ranged from 339 to 3,128, with an average of 1,574 ± 790 IU/L (normal range: <220). In addition, serum aspartate transaminase, alanine aminotransferase, and lactate dehydrogenase levels were also increased (see table 2). On biochemical analyses, acid α-1,4-glucosidase activity was normal in most male patients but was increased in some.

EKG findings were abnormal in all male patients tested (n = 17, 100%). The most frequent pathologic finding was Wolff-Parkinson-White (WPW) syndrome (six male patients) with a short PQ interval, a prolonged QRS complex, and a delta wave. Male patients with hypertrophic cardiomyopathy showed abnormally high voltage in precordial leads. In addition, giant negative T wave, third-degree atrioventricular block, atrial flutter, bradycardia, abnormal Q wave, and complete left bundle branch block were also observed. The mean age at which abnormal EKG findings were first noticed was 13 ± 5 years (n = 10). EKG also showed abnormalities in all male patients studied (n = 19); most had concentric hypertrophic cardiomyopathy with impaired left ventricular function. Seven male patients had abnormally thick interventricular septum and posterior walls. Electromyography showed small-amplitude short-duration motor unit potentials in all 10 male patients tested. In addition, fibrillation and positive sharp waves were present in three male patients, myotonic potentials at rest were noted in three individuals, and all three forms of abnormal discharges were noted in one. Nerve conduction studies were normal in all five male patients tested. Electroencephalography showed mild abnormalities in two of seven male patients; one had moderate bitemporal slowing and the other had slow α wave pattern with emergence of diffuse θ waves during sleep.

Table 3 *Immunohistochemical and histochemical characteristics of skeletal muscle in Danon disease*

Characteristic	Expression	Location
NSE	+	Vacuolar membrane
AchE	+	Vacuolar membrane
Acid phosphatase	+	Vacuolar material
Dystrophin	+	Vacuolar membrane and sarcolemma
Laminin	+	Vacuolar membrane and sarcolemma
Sarcoglycan	+	Vacuolar membrane and sarcolemma
LAMP-2	−	

NSE = nonspecific esterase; AchE = acetylcholinesterase.

Fourteen of the 20 mothers were symptomatic (70%). Four of the five asymptomatic mothers (80%) had single affected sons. *LAMP-2* gene sequences were normal in blood DNA of two asymptomatic mothers. The clinical features of 18 affected women are summarized in table 2.

It is difficult to define the age at onset in affected women because of the insidious nature of the disease. In the present study, the age at onset in the affected women was 38 ± 12 years (range, 12 to 53 years). Six women died (average age of 40 ± 7 years) due to cardiac failure or cardiac arrest.

Cardiomyopathy was evident in all 18 affected women. Two had hypertrophic cardiomyopathy and five had dilated cardiomyopathy. Permanent pacemakers were placed in three affected women; two subsequently underwent heart transplantation. Myopathy was observed in six women (33%) with mild proximal weakness in proximal limb or neck muscles. Mild mental retardation was noted in one female patient (6%).

Serum creatine kinase was increased in five female patients and ranged from 76 to 643 IU/L. EKG findings were abnormal in all female patients; findings included left ventriclular hypertrophy, first-degree atrioventricular block, sick sinus syndrome, atrial flutter, skipped beat, abnormal Q wave, and complete left bundle branch block.

Family 1 has a frame-shift mutation in exon 9b of the *LAMP-2* gene, which is predicted to affect only the LAMP-2b isoform whereas all other mutations affect both LAMP-2a and 2b isoforms. The two affected members in that family have hypertrophic cardiomyopathy and mild myopathy but no mental retardation. Both female patients are currently alive, ages 23 and 34 years. Two heterozygous women are 56 and 27 years of age and have not developed cardiac abnormalities, muscle weakness, or elevations of serum creatine kinase.

Muscle pathology. Pathologic findings in muscle are summarized in table 3. All probands showed mild to moderate variation in fiber size. Small vacuoles were seen in many fibers, which may appear as basophilic granules rather than vacuoles in hematoxylin and eosin preparations (figure 1). The granules contained acid phosphatase–positive material. In all probands, acetylcholine and nonspecific esterase activities were associated with the vacuolar membranes. Furthermore, on immunohistochemistry the vacuolar membranes stained with antibody against dystrophin, whereas LAMP-2 was completely absent in muscle from all male patients (figure 2).

On electron microscopy, pathologic findings in muscle included numerous intracytoplasmic autophagic vacuoles with glycogen particles and cytoplasmic debris. In addition, some of the vacuoles were bounded by membranes with features of the basal lamina.

Discussion. We identified 13 families, with a total of 20 male and 18 female patients, with genetically confirmed Danon disease. All 13 probands were male, whereas women were less severely affected, with later-onset cardiomyopathy. These findings suggest that Danon disease is an X-linked dominant disorder. In fact, the causative gene for Danon disease, *LAMP-2*, has been mapped to chromosome

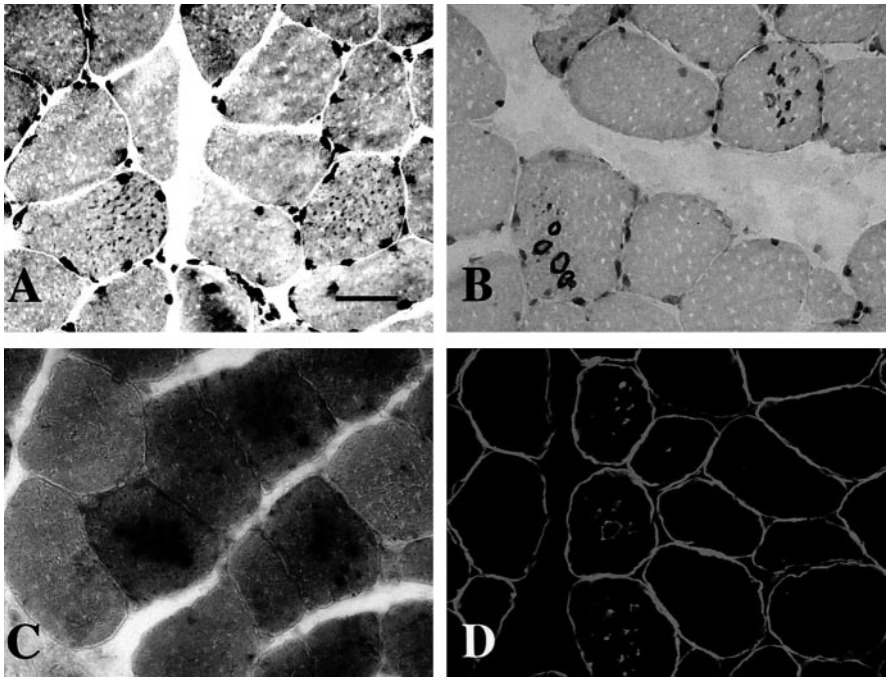

Figure 1. Histochemistry and immunohistochemistry. Transverse sections of skeletal muscle biopsy specimens from affected men with Danon disease. Several fibers with tiny basophilic intracytoplasmic vacuoles are scattered throughout (A). The vacuolar membrane has high acetylcholinesterase (B) and nonspecific esterase (C) activities. Dystrophin expressed in vacuolar membrane and sarcolemma of muscle fibers (D). (A) Hematoxylin and eosin stain. (B) Acetylcholinesterase. (C) Nonspecific esterase. (D) Immunohistochemistry with antibody against dystrophin. Bar = 40 μm.

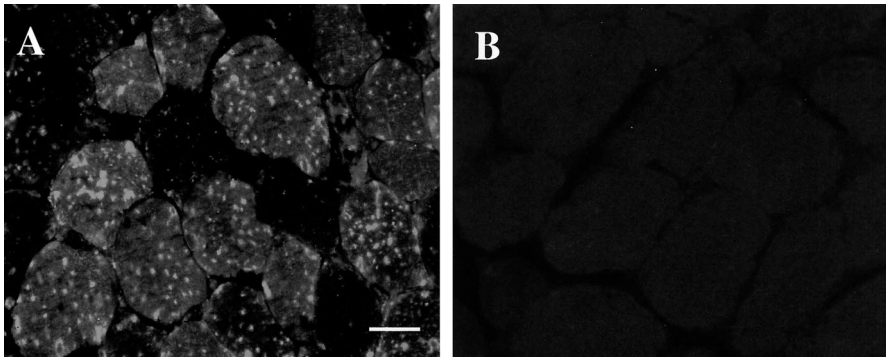

Figure 2. Immunohistochemistry. Transverse sections of skeletal muscle biopsies from control subjects (A) and affected men from Danon disease (B) were immunostained with antibody against LAMP-2. LAMP-2 is present in the control muscles, whereas it is absent in Danon disease muscles. Bar = 40 μm.

Xq24.[3] In previous studies, this disease had been defined clinically by the triad of cardiomyopathy, myopathy, and variable mental retardation. We have confirmed that this clinical triad encompasses the main clinical features of Danon disease.

Myopathy is usually mild and can be clinically silent; however, all male patients had elevated serum creatine kinase levels and myogenic changes by electromyography. Symptomatic patients typically had proximal limb weakness, which was very slowly progressive or stable. Myopathic symptoms were noted in only a few female patients and were even milder. Two female patients without overt myopathy underwent muscle biopsy; one showed no remarkable findings, but the other revealed focal vacuolation in muscle fibers.[5,11]

Electrophysiologically, in addition to myopathic units in all male patients tested, myotonic discharges were observed in three of 10 male patients; however, clinical myotonia was not evident in any patient. Therefore, myotonic discharges are not uncommon in patients with Danon disease but are observed less frequently than in patients with type II glycogenesis.[17]

Cardiac symptoms are the dominant clinical features and the most important prognostic factors, because all of the deceased patients died of cardiac failure. Histologically, cardiomyocytes have shown severe vacuolation and degeneration, including myofibrillar disruption and lipofuscin accumulation.[5,11]

Most male patients developed hypertrophic cardiomyopathy, whereas most female patients showed dilated cardiomyopathy. Previous reports described the evolution of the hypertrophic into dilated cardiomyopathy with progressive cardiac failure.[1,7] Therefore, dilated cardiomyopathy may be associated with the timing of cardiac investigation. However, other factors may determine the type of cardiomyopathy.

The WPW EKG pattern observed in Danon disease has been attributed to myocardial hypertrophy rather than to the presence of an accessory pathway.[6] However, the incidence of WPW is relatively high (6/17 male patients, 35%) in Danon disease as compared with that in other hypertrophic cardiomyopathy, such as idiopathic hypertrophic cardiomyopathy (1.5%) and familial hypertrophic cardiomyopathy (12%),[18] which suggests the presence of a specific pathomechanism for preexcitation in Danon disease rather than a simple association with cardiac hypertrophy.

Although permanent pacemakers were inserted in several patients, heart transplantation may be the most effective intervention. The only male patient who underwent heart transplantation was a 25-year-old man with hypertrophic cardiomyopathy, atrial flutter, and atrioventricular block. For 4 years after the operation, he did not have significant deteriorations except occasional rejection episodes.[11] As in men, cardiomyopathy can be fatal in female patients. This suggests that not only male patients but also women with Danon disease should be considered for heart transplantation. In addition, we suggest that asymptomatic female relatives of male patients should be investigated for cardiomyopathy and followed closely to detect early signs of a potentially life-threatening condition.

Mild mental retardation was observed in more than half of the male patients and electroencephalography showed mild abnormalities in two male patients. One male patient had decreased cerebral glucose metabolism in the cerebral cortex on PET.[13] There were no CNS manifestations and no patient showed structural brain abnormalities by CT or MRI. However, careful postmortem neuropathologic analysis is needed to further characterize the CNS involvement in Danon disease.

Muscle pathology is characterized by small basophilic vacuoles in many fibers. The vacuolar membranes show acetylcholine and nonspecific esterase activities that are very useful to distinguish Danon disease from other vacuolar myopathies including acid maltase deficiency. The positive acid phosphatase stain is also a useful though not specific diagnostic feature. However, similar vacuoles are also seen in other autophagic vacuolar myopathies including X-linked myopathy with excessive autophagy[19] and infantile autophagic vacuolar myopathy.[8] The pathognomonic finding in Danon disease is the absence of LAMP-2 immunohistochemical staining.

In *LAMP-2* knockout mice, a wider variety of organs is affected, including liver, kidney, pancreas, small intestine, thymus, and spleen, in addition to heart and skeletal muscle.[20] Therefore, other organs may also be involved in Danon disease. In fact, some male patients had hepatomegaly and had dispropor-

tionately high serum aspartate transaminase and alanine aminotransferase levels compared with creatine kinase values. Mild portal fibrosis with normal hepatocytes was seen in liver from one male patient with cardiac dysfunction.[11] However, another biopsy specimen from another male patient without cardiopathy showed sclerotic portal and central veins, nuclear vacuolization of the hepatocytes, and enlarged mitochondria with irregular cristae,[12] suggesting that liver may be primarily affected. In addition, there was platelet dysfunction and glycogen accumulation in one other patient.[9]

Human exon 9 of the *LAMP-2* gene exists in two forms, exon 9a and 9b, that are alternatively spliced and produce two isoforms, LAMP-2a and LAMP-2b. LAMP-2a is present ubiquitously, whereas LAMP-2b is expressed predominantly in heart and skeletal muscles.[15] The mutation in Family 1 affects only the LAMP-2b isoform, whereas all other mutations affect both LAMP-2a and 2b isoforms. The two affected men in Family 1 had hypertrophic cardiomyopathy and mild myopathy but no mental retardation. In this family, one affected man is alive at age 34 years and one female patient is alive at age 56 years, suggesting that isolated deficiency of the LAMP-2b isoform may be associated with a milder phenotype.

Most mothers of the probands have shown cardiac abnormalities. This finding is in sharp contrast to the broad range of clinical severity in manifesting carriers of other X-linked recessive disorders, such as Duchenne muscular dystrophy. Although we identified five asymptomatic mothers, all but one (80%) had sporadic affected sons, raising the possibility that these singleton male patients were due to spontaneous mutations. In support of this notion, two of the asymptomatic mothers did not harbor a mutation in blood DNA. All available evidence indicates that Danon disease is an X-linked dominant disease.

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